The interaction of *Trypanosoma congolense* with endothelial cells

A. HEMPHILL[1], I. FRAME[2] *and* C. A. ROSS[3]

[1] *Institute of Parasitology, University of Berne, Laenggass-Strasse 122, 3001 Berne, Switzerland*
[2] *Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK*
[3] *Centre for Tropical Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian, Edinburgh EH25 9RG, UK*

(*Received 17 January 1994; revised 11 April 1994; accepted 15 April 1994*)

SUMMARY

Factors which affect adhesion of cultured *Trypanosoma congolense* bloodstream forms to mammalian feeder cells have been examined. Using an *in vitro* binding assay, the initial events following interaction of trypanosomes with bovine aorta endothelial (BAE) cells were monitored by both light- and electron microscopy. Metabolic inhibitors and other biochemicals were incubated with either cells or parasites, to test whether any inhibited the process. Our findings suggest that adhesion of the parasites is an active process requiring metabolic energy from the trypanosomes, but not from endothelial cells. We also provide data suggesting that *T. congolense* bloodstream forms possess a lectin-like domain, localized at distinct sites on their flagellar surface, which interacts with specific carbohydrate receptors, most likely sialic acid residues, on the endothelial cell plasma membrane. We also suggest that the cytoskeletal protein actin is probably involved in this interaction.

Key words: African trypanosomes, endothelial cells, cell adhesion molecules, carbohydrates, actin.

INTRODUCTION

Trypanosoma congolense, (Broden, 1904), is one of the most important agents of African animal trypanosomiasis, and infections cause extensive losses in economic productivity throughout the continent. This parasite is pathogenic for domestic livestock, especially cattle, and infected animals show severe signs of organ damage, anaemia, weight loss, abortion and infertility (Molyneux & Ashford, 1983). Once infection has taken place, after transmission by the tsetse fly vector, rapidly dividing trypanosomes circulate within the bloodstream of mammalian hosts, protected from the immune response by their variable surface glycoprotein (VSG) coat (Vickerman, 1985; Boothroyd, 1985). In contrast to other trypanosomatids *T. congolense* does not invade tissues, like *T. brucei* or *T. evansi*, or cells, like the South American *T. cruzi*, and can be regarded as a true extracellular blood parasite.

In addition, the flagellum of *T. congolense*, unlike other trypanosomatids, does not extensively protrude from the anterior end of the cell body (Vickerman, 1969; Vickerman & Tetley, 1990). However, this organelle appears to play a key role in the cell adhesion events which occur at different life-cycle stages within the insect vector and in animal hosts. In infected animals, *T. congolense* bloodstream forms are found adhering to erythrocytes and to endothelial cells of the microvasculature through their flagellum (Banks, 1978, 1979, 1980). The direct effects of this process are not entirely understood, but the tissue surrounding the adhesion sites is damaged by release of a vascular permeabilization factor, and the subsequent action of the host's immune system (Banks, 1980).

The binding of *T. congolense* to endothelial cells has not been investigated at the molecular level. The aim of these studies was therefore to dissect the interaction between cultured trypanosomes and bovine aorta endothelial cells *in vitro*, a process which resembles the adhesion event between parasites and host cells *in vivo*. An *in vitro* adhesion assay was characterized during these experiments, where observations were made by light- and electron microscopy. Biochemical inhibition assays were performed, to investigate the reactive groups which take part in the interaction.

MATERIALS AND METHODS

If not otherwise stated, all reagents and tissue culture media, were purchased from Sigma (St Louis, Mo, USA).

Endothelial cells

Primary cultures of bovine aorta endothelial (BAE) cells were prepared as described by Gray *et al.*

(1985), and routinely maintained in Minimal Essential Medium (MEM) supplemented with 20% foetal calf serum (FCS) and 2 mM glutamine. Cultures of passages between 11 and 20 were used. BAE cells were either used unfixed, or were fixed when 95–100% confluent. Pre-fixed BAE cells were prepared by rinsing cover-slips 3 times with PBS, and incubating them for 10 min in freshly prepared 2% glutaraldehyde in PBS, 1% glutaraldehyde in PBS, or several concentrations of paraformaldehyde. Cover-slips were rinsed extensively in PBS, then stored in 0·16 M ethanolamine, pH 8·3, for at least 24 h at 4 °C in order to saturate free aldehyde groups. After fixation in 1% paraformaldehyde for 20 min at 4 °C, ethanolamine treatment was not necessary (Norgard *et al.* 1993, data not shown).

Trypanosome culture

Cultures were prepared from *T. congolense* TREU1627, a cloned derivative of a Gambian isolate (primary isolation code Kantong Kunda/77/LUMP/1794). Metacyclic derived *T. congolense* bloodstream forms (Gray *et al.* 1985) were grown at 34 °C in volumes of 6–8 ml in T-25 flasks, either axenically, or on BAE-feeder cells, in a medium modified from that of Majiwa *et al.* (1993). This consisted of a combination of RPMI 1640 Medium and Iscove's modified Dulbecco's Medium (RPMI/IMDM, 1/1) supplemented with 20% heat-inactivated (56 °C, 30 min) donor goat serum, 4 mM glutamine, 1 mM sodium pyruvate, 0·2 mM adenosine, 0·1 mM bathocuproine-sulphonate and 0·1 mM monothioglycerol. Trypanosomes were released from their feeder cells mechanically, by removing the medium, and vigorously striking the side of the flask 2–3 times. A few ml of fresh medium were added, and trypanosomes were washed from the monolayer, which remained adhered to the flask.

Adhesion assay

Immediately prior to the assay, BAE cells on cover-slips were rinsed 3 times with PBS, and once with RPMI/IMDM containing 0·1% bovine serum albumin (medium/BSA). Harvested trypanosomes were centrifuged at 800 ***g*** for 10 min at room temperature and resuspended, at a density of 5×10^5 ml, in PBS, PBS/0·1% BSA, PBS/100 mM glucose, RPMI/IMDM, medium/BSA, or trypanosome culture medium, depending on the experiments to be performed. Trypanosomes were incubated with the cell monolayers for 1 h, then the cover-slips were rinsed carefully 3 times with PBS, fixed for several hours in 1% glutaraldehyde in PBS at 4 °C, and processed for light- and electron microscopy.

The number of adherent parasites in each experiment was resolved light microscopically by randomly choosing 20 different fields at 40 times magnification on a Polyvar light microscope, counting the monolayer-bound trypanosomes, and determining the average number of adherent parasites per field. For every experiment a control incubation was performed. The numbers of trypanosomes adhering in each experiment are expressed as a percentage, in relation to the control experiment conducted on the same day with the same culture. All experiments were performed in duplicate, and the data presented are from 1 representative experiment out of 4–6 carried out.

In order to compare the number of adhering trypanosomes to fixed and unfixed BAE cell monolayers during a time-course, trypanosomes were resuspended in culture medium at 10^5 parasites/ml and incubated with the monolayers. After various time-points one cover-slip each was rinsed in PBS and fixed as described above.

Light microscopy

Glass cover-slips containing glutaraldehyde-fixed *T. congolense* bloodstream forms adhering to BAE cells were washed in distilled water, and were stained with haemotoxylin and eosin. Cover-slips were finally dehydrated in ethanol and xylene and mounted onto glass slides.

Scanning electron microscopy (SEM)

Glass cover-slips with BAE-cell monolayers and adhering *T. congolense* were fixed in PBS plus 2% glutaraldehyde for 4 h at 4 °C, were rinsed several times in PBS and treated with 1% osmium tetroxide in PBS for 1 h at 4 °C. Specimens were then dehydrated through a series of acetone (70–90–100%), and were critical-point dried. Specimens were viewed in a Jeol JSM-255 III scanning electron microscope operating at 12·5–20 kV.

Transmission electron microscopy (TEM)

Cover-slips containing bloodstream forms adhering to BAE cell monolayers were processed for thin section EM as follows. Cells were fixed in 2% glutaraldehyde in 0·1 M cacodylate buffer (Fluka Chemicals, UK), pH 7·3, for 2 h at room temperature. They were post-fixed with 1% osmium tetroxide in veronal acetate buffer, pH 7·4, for 1 h at 4 °C, followed by buffer rinses. The cover-slips were then carried through a graded series of ethanol (70–95–100%) and embedded in Epon 812 resin (Fluka Chemicals, UK). After polymerizing the resin at 65 °C for 24 h, the glass cover-slips were removed by dipping the specimen into liquid nitrogen, and the freshly exposed surfaces were embedded in Epon 812 again. Ultrathin sections

were finally cut perpendicular to the surface onto which the cells had previously been adhering, using a Diatom diamond knife on a LKB-ultramicrotome. Sections were loaded onto 200 mesh copper or nickel grids and stained with uranyl acetate and lead citrate (Smith & Croft, 1991). Specimens were viewed on a Jeol 100 CX II transmission electron microscope operating at 60–80 kV.

Treatment of live endothelial cells prior to the adhesion assay

Monolayers were treated with cycloheximide (50 μg/ml) in culture medium for 6 h at 37 °C prior to fixation. Tunicamycin, was added to the medium at 2·5 μg/ml for 4 h at 37 °C. Glycolytic inhibition of BAE cells was performed by incubating cells in PBS containing 100 mM deoxyglucose for 30 min at 37 °C. The effect of mitochondrial inhibitors was assayed by treating cells with 1–5 mM sodium azide, 10–100 nM antimycin or 1–5 μM oligomycin (all in PBS) for 20 min prior to fixation. BAE cell monolayers were also incubated for 4 h in culture medium containing either 10 μg/ml taxol, 0·5 μg/ml nocodazole, or various concentrations of cytochalasin D (10^{-5}–10^{-7} M). The effects of these drugs on the microtubule network and the actin filament system were visualized by immunofluorescence using a polyclonal rabbit anti-tubulin antibody, or a polyclonal rabbit anti-skeletal muscle actin antibody (not shown). Cover-slips were then rinsed briefly in PBS and fixed for 20 min in 1% paraformaldehyde in 100 mM cacodylate buffer, pH 7·3, at 4 °C, and rinsed several times in PBS before the adhesion assay was carried out.

Treatment of pre-fixed BAE cells

Monolayers were fixed in 1% paraformaldehyde in 100 mM cacodylate buffer, pH 7·3, at 4 °C, and were given the following treatments before the adhesion assay was carried out. (i) Periodate treatment: monolayers were subjected to different concentrations (2–20 mM) of sodium periodate in 50 mM sodium acetate, 100 mM NaCl, pH 5, at 4 °C for 30 min. Alternatively, incubations were performed in 2–20 mM sodium periodate in PBS (pH adjusted to 7) at 4 °C. Cells were washed extensively with PBS before applying the trypanosomes. (ii) Lectin binding: BAE monolayers were incubated for 45 min at 37 °C in PBS with 50 μg/ml of the following lectins: wheat germ agglutinin (WGA), specific for *N*-acetyl-glucosamine and *N*-acetyl-neuraminic acid residues, succinylated WGA (Vector Laboratories, England), specific for *N*-acetyl-glucosamine only (Monsigny *et al.* 1980), *Tetragonolobus purpurea* agglutinin (TPA), specific for fucosyl residues, Concanavalin A (Con A), specific for D-mannosyl and D-glucosyl residues, and soybean agglutinin (SBA), specific for *N*-acetyl-glactosamine. For each lectin a control incubation was conducted in the presence of 0·4 M of its corresponding inhibitory monosaccharide (Benhamou, 1989). After washing the cover-slips with PBS, the adhesion assay was performed. (iii) Neuraminidase treatment. Live and pre-fixed BAE cells were rinsed twice in PBS and once in 20 mM MES-buffer, 100 mM NaCl, 4 mM $CaCl_2$, pH 5·5. Neuraminidase Type V from *Clostridium perfringes* was added to the same buffer at a concentration of 5 U/ml, and the cells were treated with this enzyme at 37 °C for 60 min, before being washed several times with PBS. As a minor impurity the protease caseinase (0·0001 unit/mg) was present in the enzyme preparation. (iv) Poly-L-lysine and dextran sulphate: in order to investigate possible charge interactions, pre-fixed and live endothelial cells were incubated with poly-L-lysine (2–20 μg/ml) or dextran sulphate (3–30 μg/ml), for 1 h at 4 °C.

Pre-treatments of T. congolense

Freshly isolated trypanosomes were given the following treatments before performing the adhesion assay.

Pre-fixation. Freshly isolated trypanosomes were fixed by the addition of 1% glutaraldehyde or 2% paraformaldehyde to the medium for 5–10 min at 4 °C. They were washed in PBS and kept in 0·16 M ethanolamine, pH 8·3, at 4 °C overnight. After washing the cells 3 times in PBS they were resuspended in medium/BSA and assayed for endothelial cell adhesion at 10^6 trypanosomes/ml in medium BSA.

Drug treatments. Trypanosomes (10^6/ml) were incubated in PBS/0·5% BSA in the presence of 10–100 mM deoxyglucose for 20 min at room temperature. Sodium azide (1–20 mM), antimycin (1–100 nM), oligomycin (1–5 μM) were used as mitochondrial inhibitors. Parasite numbers were then adjusted to five times 10^5/ml and were immediately used for the adhesion assay. Cycloheximide treatment was carried out at 30 μg/ml for 4 h at 34 °C in culture medium. Tunicamycin was added to 10^6/ml of trypanosomes at 0·5 μg/ml and cells were incubated for 60 min at 34 °C prior to the adhesion assay. For the cytochalasin D treatment, 10^6 parasites/ml were incubated in 10^{-7}, 10^{-6} and 10^{-5} M cytochalasin D for 20 min at 34 °C in culture medium, before applying them to the monolayers at 10^5parasites/ml. Taxol was used at 1–10 μg/ml, and nocodazole at 0·1–1 μg/ml in culture medium for 6 h at 34 °C.

Surface protease treatment. Freshly isolated trypanosomes (10^6/ml) were spun down and resuspended in medium without serum. Trypsin protease type VI and pronase (Serva, GB) were both added to

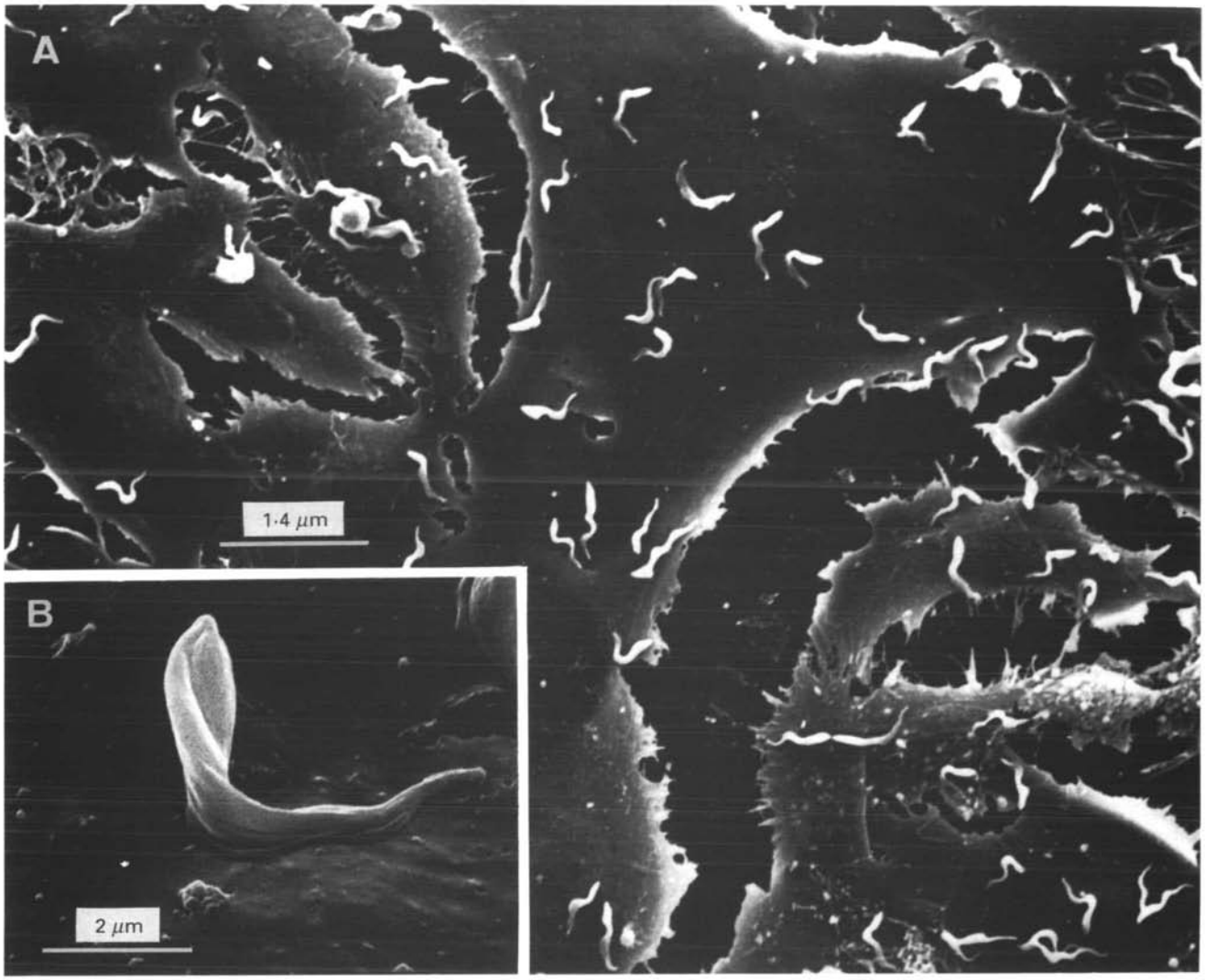

Fig. 1. SEM of *Trypanosoma congolense* adherent to bovine aorta endothelial (BAE) cells. Monolayers were grown on glass cover-slips, incubated with the parasites and processed for SEM as described in the Materials and Methods section. (A) Low magnification view demonstrating the adherence of the parasites onto the endothelial cell surface. (B) Single adherent trypanosome showing that the flagellum is the only part of the parasite directly in contact with the BAE cell surface.

2–10 μg/ml (Reinwald, 1985). After an incubation at 28 °C for 20 min, the cells were put on ice. In the case of trypsin the reaction was stopped by the addition of 80 μg/ml of soybean trypsin inhibitor type I-S. The cells were washed twice with ice-cold culture medium, were counted and immediately used for the binding assay in culture medium.

RESULTS

Morphology and ultrastructural observations

Scanning electron microscopy of adherent *T. congolense* bloodstream forms (Fig. 1A) shows that the parasites adhered to the cell surface membrane of BAE cells and not to the cover-slip underneath. Trypanosomes were bound to the endothelial cell surface exclusively by the anterior two thirds of the flagellum (Fig. 1B). These findings were confirmed in sections cut perpendicular to the plane of the cover-slip and viewed by TEM (Fig. 2). The microtubule skeleton (MT), a single layer of microtubules forming the cage round the cell body, was clearly visible, as well as the paraflagellar rod (PFR), oriented towards the trypanosome cell body, and the microtubular axoneme (Ax), facing the endothelial cell membrane. The flagellum adhered at approximately 45° with respect to the medial axis laid through the axoneme and the PFR.

Longitudinal sections through the adhering flagellum (Fig. 3) suggest that the initial attachment was not mediated through an extensively wide area on the flagellum, although the two membranes seemed to be crossbridged closely to each other over long stretches. The trypanosomal surface components directly interacting with the endothelial cell membrane appeared to be situated along a thin line on the anterior flagellum.

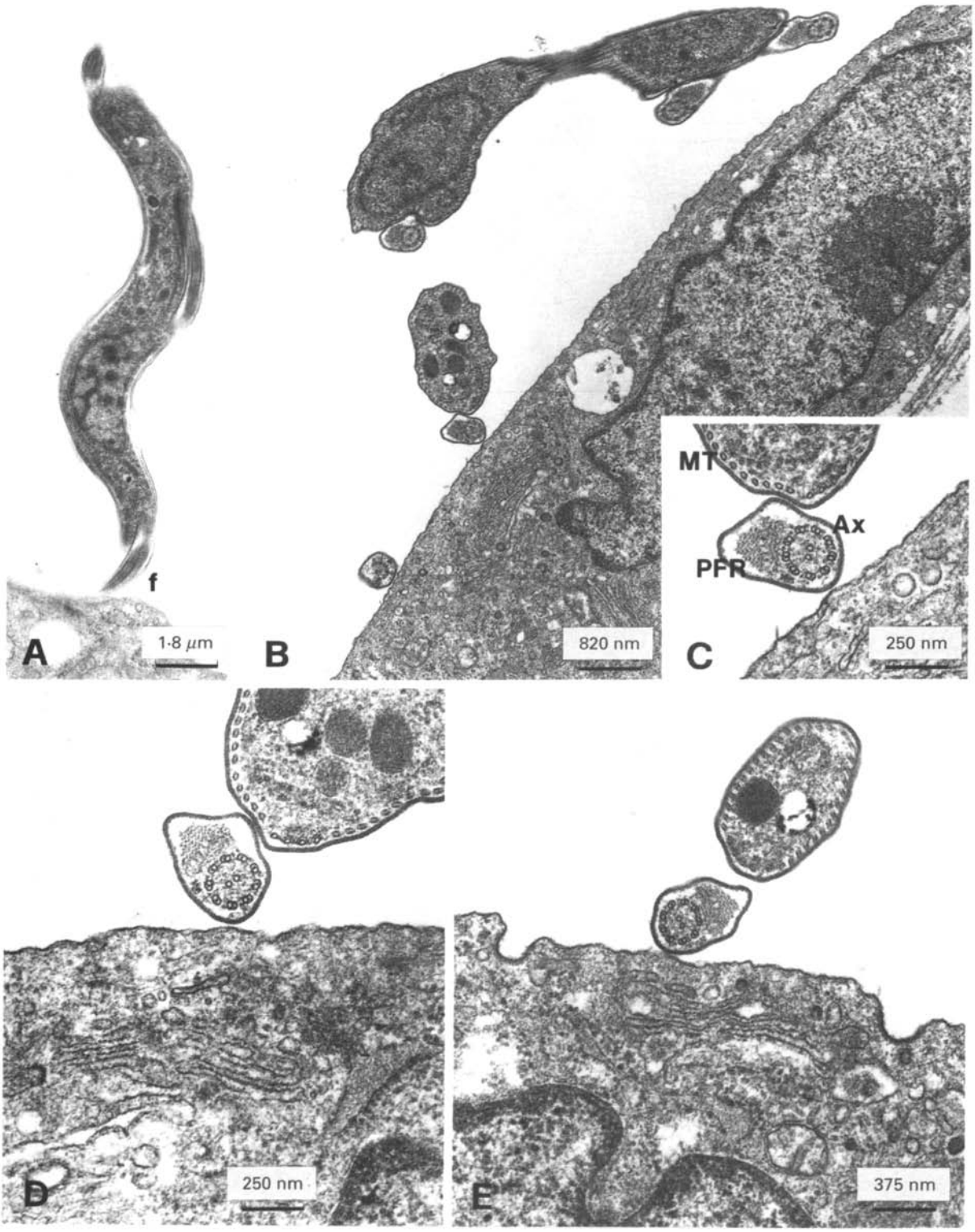

Fig. 2. TEM of adherent *Trypanosoma congolense*. (A)–(C) Micrographs of trypanosomes adhering onto live BAE cells. Longitudinal section (A) and cross-section (B) at lower magnification. f, Flagellum. (C) Higher magnification view of (B). MT, microtubules; PFR, paraflagellar rod; Ax, axoneme. (D) Trypanosomes adhering onto BAE cells pre-fixed in 2% paraformaldehyde. (E) BAE cells pre-fixed in 2% glutaraldehyde.

The effect of pre-fixation and metabolic inhibition of BAE-cells on their association with T. congolense

Initial attachment of *T. congolense* bloodstream forms to feeder cells was not altered by pre-fixation of BAE cells by formaldehyde or glutaraldehyde (data not shown). Electron microscopy showed no ultrastructural alterations compared to control cultures (Fig. 2D and E). To obtain information on the time dependence of the adhesion process, parasites were incubated at 34 °C with fixed and unfixed feeder cells, and the number of adherent trypano-

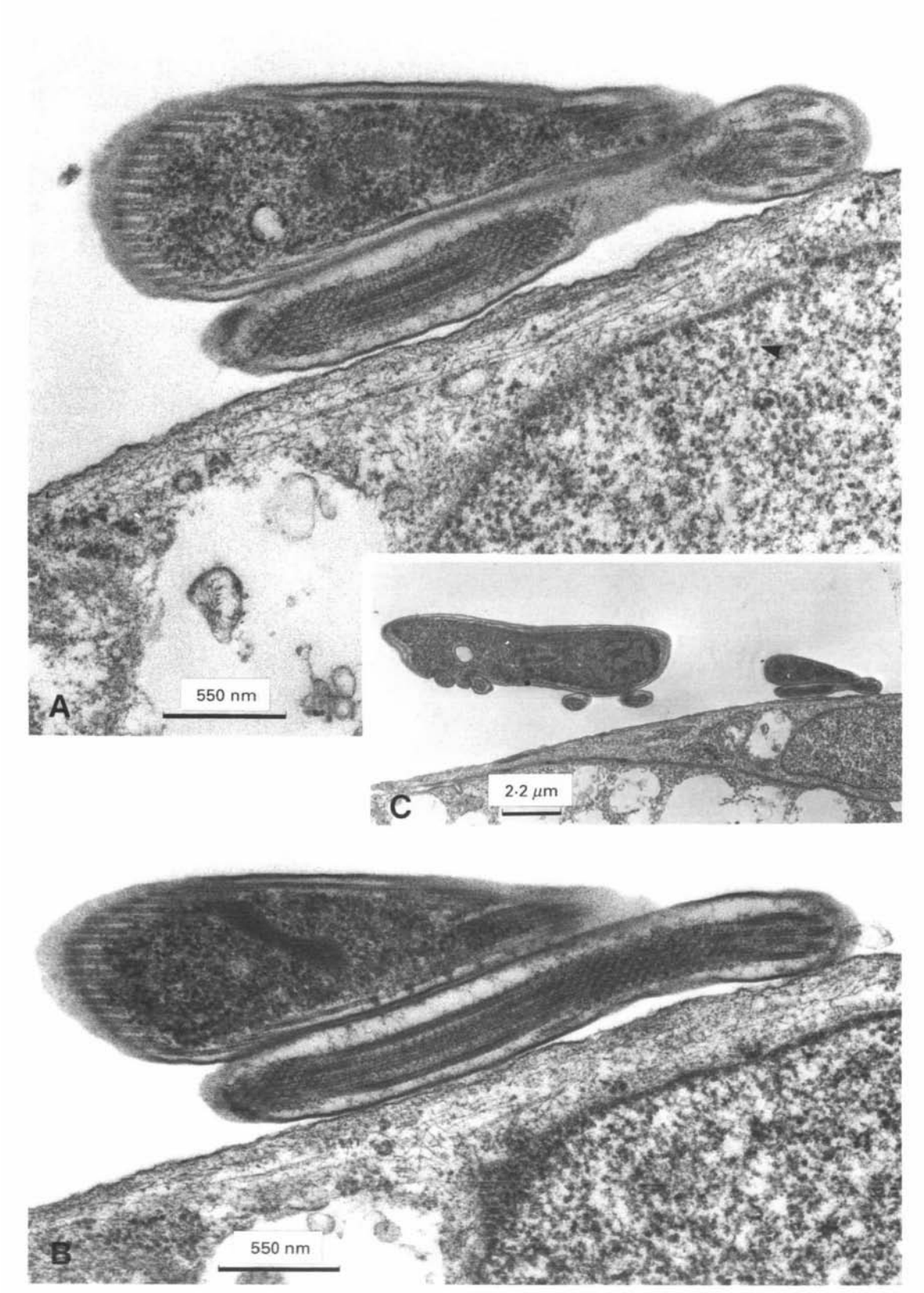

Fig. 3. TEM of adherent trypanosomes. (A) and (B) Two longitudinal serial sections through a flagellum adherent to the endothelial cell surface. Note that the site of intimate contact between the flagellar and endothelial membrane appears to be constituted by a thin line along the anterior flagellar surface (see also Fig. 2). (C) Lower magnification view of (A).

somes counted after different time-points (Fig. 4). There was no difference in the adhesion kinetics of *T. congolense* to fixed and unfixed cells within the first 60 min. After approximately 120 min, the number of trypanosomes adherent to fixed monolayers reached saturation point, while the numbers

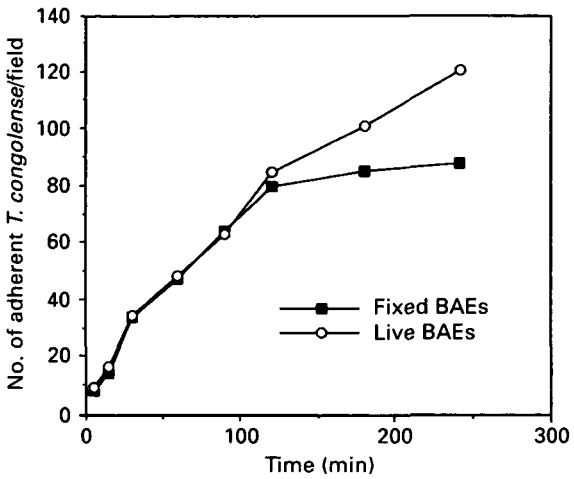

Fig. 4. Comparison of adhesion of *Trypanosoma congolense* bloodstream forms on live and pre-fixed (2% glutaraldehyde) BAE cell monolayers during a time-course of 4 h.

counted adhering to live BAE cells showed a further increase. The number of parasites adhered to unfixed cells continued to rise over 48 h of incubation, due to their multiplication, whereas trypanosomes on fixed cells scarcely divided during this time (data not shown).

Pre-treatment of BAE cells with glycolytic and mitochondrial inhibitors had no effect on adhesion of the parasites, nor had inhibition of protein synthesis with cycloheximide (data not shown). Cytoskeletal inhibitors which alter the microtubule system (taxol and nocodazole) or actin microfilaments (cytochalasin D) had no effect. Since initial adhesion was apparently independent of target cell metabolism, subsequent assays were carried out using pre-fixed BAE cells.

The involvement of endothelial cell surface carbohydrates in T. congolense *adhesion*

Both pre-treatment of BAE cells with periodate, and the pH at which this treatment was carried out, had a dramatic effect on the adherence of trypanosomes to the monolayer (Fig. 5). At 20 mM periodate, and at pH 5, adherence of trypanosomes to the monolayer was only 3·5% of that on control cells, although with a milder treatment of 2 mM periodate, adherence on treated cells was greater than 50% of the control. The reduction in adherence after periodate treatment was less marked at neutral pH.

BAE cells were also incubated with different lectins before trypanosomes were added. Con A, SBA and TPA had no effect on subsequent adhesion of trypanosomes, but prior incubation of cells with wheat germ agglutinin (WGA) inhibited parasite attachment by 60% (Fig. 5). Since WGA recognizes both *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid) residues, further experiments were carried out to determine if the adhesion process involved only one, or both sugar residues. Inhibition was not observed when cells were incubated with WGA in the presence of 0·4 M *N*-acetylglucosamine, or with succinylated WGA, which has no binding specificity for *N*-acetylneuraminic acid. The possible involvement of sialic acid residues in mediating the contact between trypanosomes and BAE cells was confirmed when neuraminidase-treated BAE monolayers showed a 95% reduction in trypanosome adhesion. In contrast, treatment with tunicamycin, an inhibitor of *N*-glycosylation, showed no effect.

Incubation with a polycation, poly-L-lysine, or with polyanionic dextran sulphate before addition of trypanosomes had no effect, showing that adherence was not based solely on charge interactions. When poly-L-lysine or dextran sulphate were present during the adhesion assay, over 60% of trypanosomes were lysed (data not shown).

The effects of pre-treatment of trypanosomes

Pre-fixed *T. congolense* bloodstream forms had completely lost their ability to adhere to the endothelial cell surface (Fig. 6). This suggested that adhesion depended on parasite metabolism, and/or that epitopes involved in the adhesion process were denatured or masked by fixation.

In a further series of experiments trypanosomes were resuspended in various buffers and media and were then allowed to adhere to BAE cells. No substantial difference in the number of adhered trypanosomes was found when parasites were resuspended in culture medium (containing 20% donor goat serum), medium/BSA, PBS/100 mM glucose, or PBS/0·5% BSA. Incubations in PBS alone resulted in a 43% reduction of adherent parasites. Carrying out the binding assay at 4 °C, or inhibiting glycolysis by incubating trypanosomes with 2-deoxyglucose, each reduced adhesion to 50% of a control culture. However, mitochondrial inhibitors such as sodium azide, antimycin and oligomycin had no effect on the number of adherent parasites.

Alteration of the parasite cell surface by pre-incubating trypanosomes with cycloheximide significantly decreased adherence. An even larger inhibition was observed when the surface of bloodstream forms was altered by protease treatment using trypsin and pronase (Fig. 6). This suggested the destruction of receptors necessary for parasite adhesion to BAE cells. Protease digestions at higher concentrations and temperatures led to considerable loss of viability of parasites (data not shown).

Parasites were incubated with several drugs which affect the function of the cytoskeleton, to investigate whether it was involved in mammalian cell interaction. Neither taxol, which enhances and stabilizes microtubules, nor nocodazole, a microtubule depolymerizing drug had any effect on adhesion of

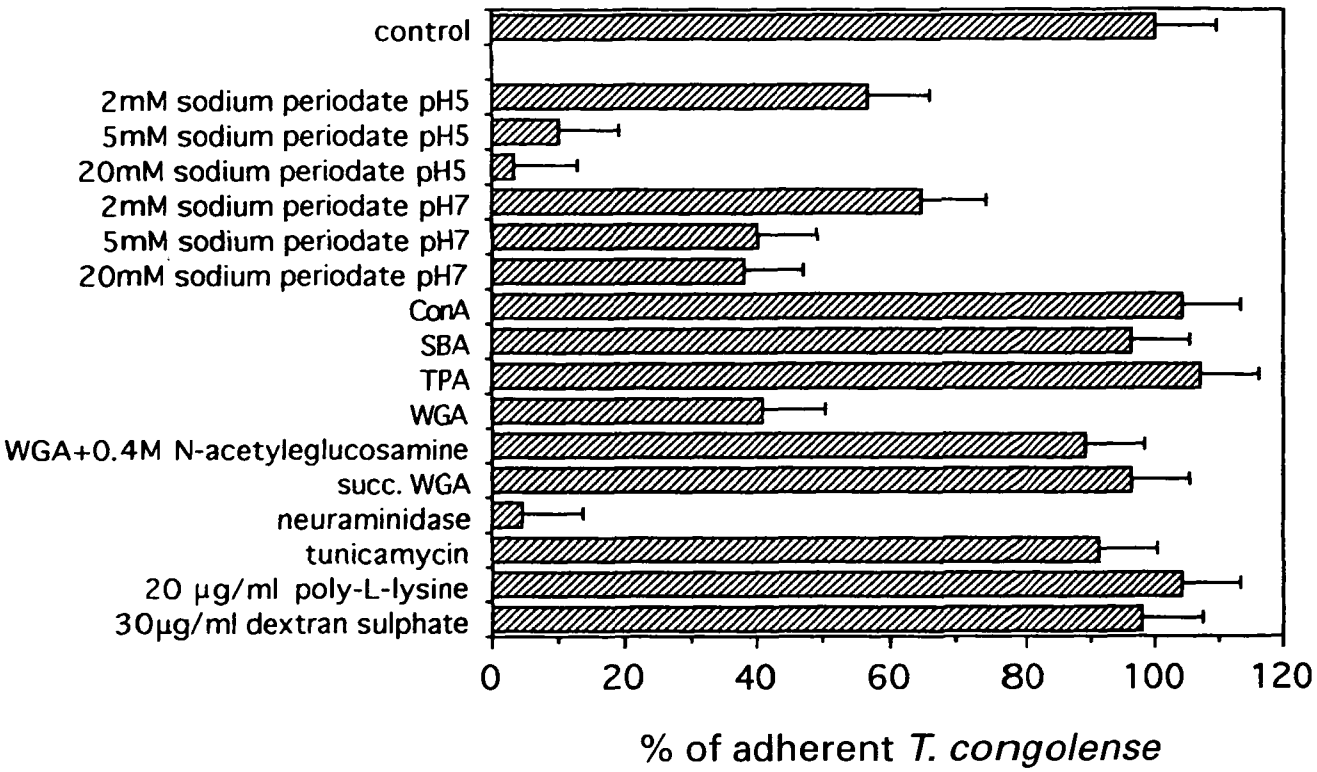

Fig. 5. Effects of treatments affecting endothelial cell surface carbohydrates on the efficiency of trypanosome adhesion. After the various treatments monolayers were fixed and incubated with the parasites as described.

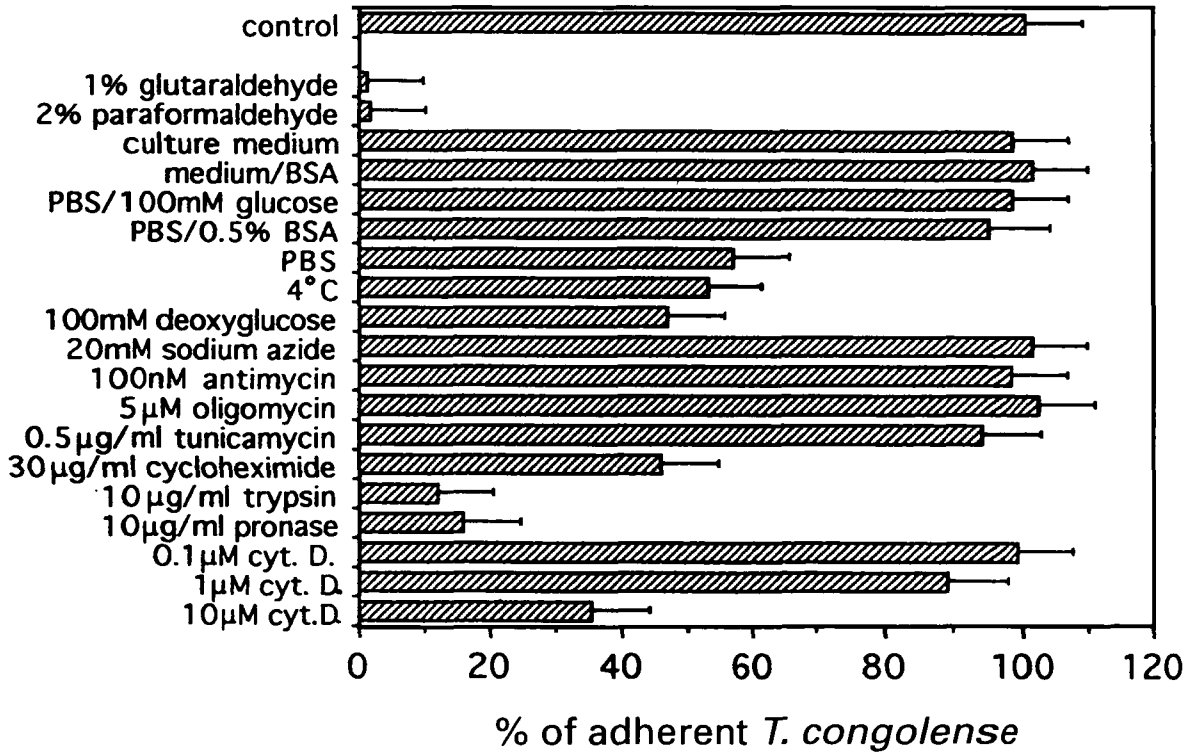

Fig. 6. Effects of treatments of *Trypanosoma congolense* bloodstream forms prior to performing the adhesion assay.

trypanosomes to BAE cells (data not shown). Treatment with 10^{-5} M cytochalasin D, a drug which specifically interacts with actin, resulted in a marked decrease in the number of adherent trypanosomes. Attempts, however, to label *T. congolense* actin with a panel of several anti-actin antibodies, or with rhodamine-coupled phalloidin, have not been successful.

DISCUSSION

In this paper the *in vitro* interaction between *T. congolense* bloodstream forms and bovine aorta endothelial cells was investigated. Observations using TEM and SEM show that *T. congolense* parasites adhere to feeder cells by a specific area in the anterior two thirds of the flagellum. Only a minor part of the flagellar surface appears to be directly involved, since the two membranes are in intimate contact with each other exclusively by a small area of the trypanosomal flagellum. These findings, and the fact that parasites can be released from the feeder cells mechanically, indicate that the interaction is rather weak. Banks (1978) studied the distribution of *T. congolense* in infected rats and rabbits, and found that trypanosomes attached by their anterior end to the walls of blood vessels. In addition, the microcirculation contained 4–1400 times as many trypanosomes than other vessels. This number could reflect the relationship between adhesion and blood pressure, since in smaller vessels the pressure generated by the blood flow is much lower than in larger vessels. *T. congolense* has also been shown to prefer the vessels of some organs over others (Losos *et al.* 1973; Kaliner, 1974), and receptor–ligand interaction during initial attachment may contribute to this preference. The EM observations also point in this direction, since the interaction is mediated solely by the anterior part of the flagellum in direct contact with the endothelial cell surface, never by the parasite cell body, and since a distinct geometric pattern of attachment is maintained in all parasites.

The adhesion of *T. congolense in vivo* to cells lining the walls of blood vessels also occurs when parasites are cultured with endothelial cells *in vitro*. Other pathogenic trypanosome species, such as *T. brucei* and *T. evansi*, do not attach to feeder cells in this way, although they can cluster in intracellular spaces. It was possible, therefore, to use an *in vitro* assay whereby endothelial monolayers grown on coverslips were provided as an adhesion matrix. Different fixation regimes had no effect on the subsequent rate of attachment to cells, although trypanosomes adhered to fixed cells did not divide and increase in number like those on unfixed cells. This suggests that BAE cells release certain factors essential for multiplication of the parasite. Banks (1979) reported that *T. congolense* adhered to glutaraldehyde-fixed and unfixed erythrocytes, and to the microvasculature, in a similar manner. Fixation of target cells has also been used in studies on the attachment of *T. cruzi* to fibroblasts and MDCK-cells (Schenkman, Robbins & Nussenzweig, 1991*a*; Schenkman, Diaz & Nussenzweig, 1991*b*), and to rat heart myoblasts (Villata *et al.* 1993). These papers suggested that adherence of *T. cruzi* trypomastigotes, which precedes cell invasion, was mediated by specific receptors on target cells at restricted cell surface domains. In contrast to these findings, our observations were that *T. congolense* did not show a preference for any specialized cell surface domain.

Recognition of specific receptor–ligand interactions, usually mediated by cell surface adhesive molecules, is a very early step in microbial colonization and pathogenesis. In many cell types, carbohydrates are known to be crucially involved in cell–cell interactions. Cell surface adhesive molecules include glycolipids (Karlsson *et al.* 1992), glycosaminoglycans (Lander, 1993), and glycoproteins (Hoffman, 1992; Oebrink, 1993). Modifications carried out on cell surface carbohydrates, therefore, are likely to influence the adhesive properties of cell surfaces. Periodate oxidation at acid pH has long been used as a tool to cleave carbohydrate residues (Woodward, Young & Bloodgood, 1985). The results presented here after the use of a whole range of periodate concentrations at low and neutral pH, show that carbohydrates are likely to be involved in *T. congolense* binding to endothelial cells. The possibility, however, that other surface membrane components are also affected by this treatment, cannot be ruled out. Further evidence of a role for carbohydrate receptors in adhesion has been obtained by the use of lectins. Wheat germ agglutinin specifically inhibits trypanosome attachment, in a manner which points towards a role for sialic acid residues in this process. In addition, neuraminidase, which specifically cleaves terminal sialic acid residues, considerably reduced the number of trypanosomes which attached to BAE cells during the adhesion assay.

Modification of the trypanosome surface was also attempted to identify parasite components which affected adhesion. Trypanosomes pre-fixed with aldehydes were no longer able to bind to endothelial cells, indicating denaturation or masking of the molecules involved. Both inhibition of protein synthesis prior to adhesion, and protease treatment of trypanosomes before the adhesion assay, reduced the number of adherent parasites. From these results it can be concluded that sialic acid residues on the endothelial plasma membrane might comprise, or at least be a significant component of, a receptor which is recognized by a specific, lectin-like trypanosome surface molecule.

This lectin-like surface protein, located at the anterior part of the flagellum and with a specialized function, is rather unlikely to be a part of the variable surface glycoprotein (VSG) coat. Firstly, the binding of trypanosomes is restricted exclusively to the anterior two thirds of the flagellum, which suggests that a non-VSG component of the *T. congolense* plasma membrane constitutes the receptor. Secondly, there is evidence that non-variable glycoproteins, as well as parts of the VSGs, are cleaved during the exposure of live *T. brucei* bloodstream forms to trypsin (Frommel, Seyfang & Balber, 1988).

The reduction in adherence after protease treatment of trypanosomes in these studies suggests a similar occurrence in *T. congolense*. Thirdly, it is interesting to note that attachment of *T. congolense* to red blood cells appeared to be mediated by sialic acid residues on the erythrocyte surface, and could be abolished by protease treatment (Banks, 1979). This interaction, however, could be inhibited by poly-L-lysine, which saturates the negatively charged sialic acid residues on the erythrocyte surface. In our experiments, neither poly-L-lysine nor dextran sulphate had any effect on trypanosome attachment to BAE cells.

Initial observations indicated that trypanosome attachment occurred independently of a functional target cell metabolism (data not shown). Experiments were then carried out to find if the attachment process required metabolic activity on the part of the parasite. Inhibition of glycolysis resulted in decreased efficiency of adhesion, suggesting that trypanosomes expend metabolic energy in their interaction with endothelial cells. Similar results were obtained in studies of South American trypanosomes and their attachment to mammalian cells (Schenkman *et al.* 1991*a*).

The cytoskeleton in most eukaryotic cells is involved in cellular events such as cell motility and surface receptor movements (Geiger, 1989). In trypanosomes, the cytoskeleton is mainly microtubule-based (Angelopoulos, 1970; Hemphill, Lawson & Seebeck 1991*a*; Hemphill, Seebeck & Lawson, 1991 b), and the presence of actin in African trypanosomes has not yet been conclusively demonstrated (BenAmar *et al.* 1988; Seebeck, Hemphill & Lawson, 1990). However, the genes coding for actin of *T. brucei* have been sequenced, and it has been shown that they are constitutively transcribed in both bloodstream and insect forms (BenAmar *et al.* 1988). In this study, parasites were treated with drugs which would specifically affect microtubules and actin filaments. Taxol and nocodazole, which are microtubule active drugs in higher eukaryotic cells, had no effect on adhesion, while cytochalasin D, a drug specifically destroying the organization of actin filaments (Cooper, 1987) did have an inhibitory effect. No specialized filamentous structures, however, could be seen by TEM. There remains the possibility that actin could form small filaments not visible by our methodology.

In conclusion, our evidence indicates that the interaction between these two cell types is mediated by certain carbohydrate receptors present on the endothelial cell surface, and one or several ligands, most likely proteins or glycoproteins, on the trypanosome, whose functional activities are affected by proteases and protein synthesis inhibitors. These ligands must be present at distinct localizations near the anterior end of the flagellum. Our investigations provide the necessary background for further studies on interactions between *T. congolense* and endothelial cells which should lead to the identification of the molecules involved. The significance and the functional importance of this adhesion is yet to be explained, but a more thorough exploration of this process will contribute to a better understanding of the pathogenesis of this parasite.

It is a pleasure to acknowledge Simon L. Croft (Department of Medical Parasitology, LSH & TM) for his encouraging support, and George Towey and Maria McCrossan (EM Unit, LSH & TM) for their technical help in electron microscopy. We thank Thomas Seebeck (University of Bern, Switzerland) for providing us with taxol and with antibodies directed against tubulin and actin, as well as Robert Harris (Department of Medical Parasitology, LSH & TM), and Norbert Mueller (Institute of Parasitology, University of Berne, Switzerland), for their helpful discussions and comments on the manuscript. This work was supported by a fellowship from the Swiss National Foundation, a stipend from the Bernese Cancer League to A.H., and the Overseas Development Administration.

REFERENCES

ANGELOPOULOS, E. (1970). Pellicular microtubules in the family *Trypanosomatidae*. *Journal of Protozoology* **17**, 39–51.

BANKS, K. L. (1978). Binding of *Trypanosoma congolense* to the walls of the small blood vessels. *Journal of Protozoology* **25**, 241–5.

BANKS, K. L. (1979). *In vitro* binding of *Trypanosoma congolense* to erythrocytes. *Journal of Protozoology* **26**, 103–8.

BANKS, K. L. (1980). Injury induced by *Trypanosoma congolense* adhesion to cell membranes. *Journal of Parasitology* **66**, 34–7.

BENAMAR, M. F. A., PAYS, A., TEBABI, P., DERO, B., SEEBECK, T., STEINERT, M. & PAYS, E. (1988). Structure and transcription of the actin gene in *Trypanosoma brucei*. *Molecular and Cellular Biology* **8**, 2166–7.

BENHAMOU, N. (1989). Preparation and application of lectin-gold complexes. In *Colloidal Gold: Principles, Methods and Applications*, vol. 1 (ed. Hayat, M. A.), pp. 95–143. London: Academic Press.

BOOTHROYD, J. C. (1985). Antigenic variation in African trypanosomes. *Annual Review in Microbiology* **39**, 475–502.

BRODEN, A. (1904). Les infections a trypanosomes au Congo chez l'homme et les animaux. *Bulletin de la Société belge d'études coloniales, Bruxelles*.

COOPER, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *Journal of Cell Biology* **105**, 1473–8.

FROMMEL, T. O., SEYFANG, S. & BALBER, A. E. (1988). *Trypanosoma brucei ssp.*: Cleavage of variant specific and common glycoproteins during the exposure of live cells to trypsin. *Experimental Parasitology* **66**, 213–24.

GEIGER, B. (1989). Cytoskeleton associated cell-contacts. *Current Opinion in Cell Biology* **1**, 103–9.

GRAY, M. A., ROSS, C. A., TAYLOR, M. A., TETLEY, L. & LUCKINS, A. G. (1985). *In vitro* cultivation of *Trypanosoma congolense*: the production of infective

forms of metacyclic trypanosomes cultured on bovine aorta endothelial cell monolayers. *Acta Tropica* **42**, 99–110.

HEMPHILL, A., LAWSON, D. & SEEBECK, T. (1991*a*). The cytoskeletal architecture of *Trypanosoma brucei*. *Journal of Parasitology* **77**, 603–12.

HEMPHILL, A., SEEBECK, T. & LAWSON, D. (1991*b*). The *Trypanosoma brucei* cytoskeleton: ultrastructure and localization of microtubule associated and spectrin like proteins using quick-freeze, deep-etch immunogold electron microscopy. *Journal of Structural Biology* **107**, 211–20.

HOFFMAN, S. (1992). Assays of cell adhesion. In *Cell–Cell Interactions. A Practical Approach.* (ed. Rickwood, D. & Hames, B. D.), pp. 1–29. Oxford: IRL Press.

KALINER, G. (1974). *Trypanosoma congolense* II. Histochemical findings in experimentally infected cattle. *Experimental Parasitology* **86**, 20–6.

KARLSSON, K. A., MILH, M. A., ANGGSTROEM, J., BERGSTROEM, J., DEZFOOLIAN, H., LANNE, B., LEONARDSON, I. & TENEBERG, S. (1992). Membrane proximity and internal binding in the microbial recognition of host cell glycolipids: a conceptual discussion. In *Molecular Recognition in Host–Parasite Interactions. FEMS Symposium No. 61*, (ed. Korhonen, T. K., Tapani, H. & Maekelae, P. H.), pp. 115–132. New York. Plenum Press.

LANDER, A. D. (1993). Proteoglycans. In *Guidebook to Extracellular Matrix and Adhesion Proteins*, (ed. Kreis, T. & Vale, R.), pp. 12–16. Oxford: Oxford University Press.

LOSOS, G., PARIS, J., WILSON, A. J. & DAR, F. K. (1973). Distribution of *Trypanosoma congolense* in tissues of cattle. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **67**, 278.

MAJIWA, P. A. O., MAINA, M., WAITWUBI, J. N., MIHAK, S. & ZWEYGARTH, E. (1993). *Trypanosoma* (*Nannomonas*) *congolense*: molecular characterization of a new genotype from Tsavo, Kenya. *Parasitology* **106**, 151–62.

MOLYNEUX, D. H. & ASHFORD, R. W. (1983). African animal trypanosomiasis. In *The Biology of Trypanosomes and Leishmania Parasites of Man and Domestic Animals.* (ed. Taylor and Francis). London: Academic Press.

MONSIGNY, M., ROCHE, A. C., SENE, C., MAGET-DANA, R. & DELMOTTE, F. (1980). Sugar-Lectin interactions: How does wheat-germ agglutinin bind sialoglycoconjugates? *European Journal of Biochemistry* **104**, 147–53.

NORGARD, K. E., HAN, H., POWELL, L., KRIEGLER, M., VARKI, A. & VARKI, N. M. (1993). Enhanced interaction of L-selectin with the high endothelial venule ligand via selectively oxidized sialic acids. *Proceedings of the National Academy of Sciences, USA* **90**, 1068–72.

OEBRINK, B. (1993). Cell adhesion and cell-cell contact proteins. In *Guidebook to Extracellular Matrix and Adhesion Proteins* (ed. Kreis, T. & Vale, R.), pp. 109–114. Oxford: Oxford University Press.

REINWALD, E. (1985). Role of carbohydrates within variant surface glycoproteins of *Trypanosoma congolense*. *European Journal of Biochemistry* **151**, 385–91.

SCHENKMAN, S., ROBBINS, E. S. & NUSSENZWEIG, V. (1991*a*). Attachment of *Trypanosoma cruzi* requires parasite energy and invasion can be independent of the target cell cytoskeleton. *Infection and Immunity* **59**, 645–54.

SCHENKMAN, S., DIAZ, C. & NUSSENZWEIG, V. (1991*b*). Attachment of *Trypanosoma cruzi* trypomastigotes to receptors at restricted cell surface domains. *Experimental Parasitology* **72**, 76–86.

SEEBECK, T., HEMPHILL, A. & LAWSON, D. (1990). The cytoskeleton of trypanosomes. *Parasitology Today* **6**, 49–52.

SMITH, M. & CROFT, S. L. (1991). Embedding and thin section preparation. In *Electron Microscopy in Biology. A Practical Approach* (ed. Rickwood, D. & Harris, B. D.), pp. 17–37. Oxford: IRL Press.

VICKERMAN, K. (1985). The developmental cycles and biology of pathogenic trypanosomes. *British Medical Bulletin* **41**, 105–14.

VICKERMAN, K. (1969). The fine structure of *Trypanosoma congolense* in its bloodstream phase. *Journal of Protozoology* **16**, 54–69.

VICKERMAN, K. & TETLEY, L. (1990). Flagellar surfaces of parasitic protozoa and their role in attachment. In *Ciliary and Flagellar Membranes*, (ed. Bloodgood, J. R.), pp. 267–304. New York: Plenum Press.

VILLATA, F., RUIZ-RUANO, A., VALENTINE, A. A. & LIMA, M. F. (1993). Purification of a 74 kD glycoprotein from heart myoblasts that inhibits binding and entry of *T. cruzi* into heart cells. *Molecular and Biochemical Parasitology* **61**, 217–30.

WOODWARD, M. P., YOUNG, W. W. & BLOODGOOD, J. R. (1985). Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *Journal of Immunological Methods* **78**, 143–53.